RESEARCH ARTICLE

https://doi.org/10.21608/FPMPEB.2025.437804

Vol. 2, Issue 1 /No4/2025

Differential Expression of HLA-G and NF-κB Diagnostic and Prognostic Implications

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Abstract

Immune evasion is a key player in breast cancer (BC) progression. This study examines the expression patterns of immunomodulatory molecules HLA-G and NF-κB in breast cancer patients, comparing them with benign breast tumor patients and healthy controls.

A total of 45 females were investigated and categorized into three groups: Control (n=10), Benign breast (n=10), and Malignant breast cancer patients (n=25; stage II or higher). Routine laboratory investigations, pathological evaluations, hormone receptor status assessments, and ELISA-based quantification of serum, tissue homogenate, and PBMC lysate levels of HLA-G and NF-κB were performed.

Liver and renal function tests revealed no significant difference across groups. WBC count was elevated in the malignant group, suggesting a systemic inflammatory response. The most common histological subtype in the malignant group was invasive ductal carcinoma (84%), with most cases being Grade II and stages II or III. ER and PR positivity was detected in 72% of malignant cases. Tissue HLA-G and NF-κB levels were significantly higher in malignant versus benign tissues ($p < 0.001$ and $p = 0.003$, respectively). Serum sHLA-G was significantly elevated in the malignant group compared to controls ($p = 0.041$). NF-κB expression in PBMCs was markedly upregulated in the malignant group ($p < 0.001$). No significant correlations were found between sHLA-G levels and clinicopathological parameters, although higher levels were observed in patients with hormone receptor positivity and lobular carcinoma.

Elevated HLA-G and NF-κB levels in breast cancer patients underscore their potential as immune escape markers and prognostic indicators. Their differential expression across serum, tissue, and PBMCs confirmed tumor-host immune interactions and may aid in refining immunotherapeutic strategies.

Keywords: HLA-G; NF-κB; Breast Cancer.

1. Introduction

Breast cancer is a complex and heterogeneous disease whose classification has been significantly improved in recent years through the development of gene expression signatures [1]. However, significant disparity in clinical outcome remains within different disease entities, leading investigators to continue searching for more refinement. In this respect, Clinical studies utilizing prognostic and predictive gene expression signatures have demonstrated that the strength of the immune response as strongly associated with cancer progression and patient outcomes [2]

Breast cancer is frequently initiated by genetic and epigenetic changes that may alter the functions of the mammary gland epithelial cells [3]. Furthermore, inherited genetic mutations and epigenetic modifications cause premalignant transformation of mammary cells [4]. While immunosurveillance can restrain or eradicate cancer cells and inhibit tumor development, immune preference and evasion promote advanced breast tumors [5]. This progression is often accompanied by protumorigenic inflammation, which raises immune avoidance and weakens effective immunosurveillance [6].

In this context, cancer cells display tumor-associated antigens coded by mutated or deregulated genes. Once presented by classical MHC class I molecules, these antigens would be

recognized and eradicated by the host immune system [7]. Nonetheless, the presence of a competent immune system, neoplastic cells may still grow and progress to very aggressive malignant lesions through tumor immunoediting process [8].

Jiang et al; (2015) suggested a model by which breast cancer cells evade immunosurveillance via major immunosuppressive cells including regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSCs) [9]. These cells are recruited and activated by proinflammatory mediators produced in the breast tumor microenvironment. The activated Treg and MDSCs would suppress cytotoxic T lymphocyte (CTL) and natural killer (NK) cells which are considered as potent antitumor effector cells [10]. Breast cancer cells also produce soluble factors such as Indoleamine 2,3-dioxygenase, (IDO), IL-10, tumor growth factor β (TGF-β) and soluble Major Histocompatibility Complex class I related chain A (sMICA) to suppress the activity of cytotoxic T lymphocytes (CTL) and NK cells [11]. Furthermore, breast cancer cells modulate the expression levels of apoptosis-associated intracellular proteins [proteinase inhibitor9 (PI-9), apoptosis regulator protein (BAX-a) and Survivin [12]. Cancer cells also modulate the immune recognition- or activation-associated membranous proteins [MHC class I polypeptide-related sequence B (MICB), human leukocyte antigen (HLA)-class I, TNF-related apoptosis-inducing ligand (TRAIL)-R1, TRAIL-R2, HLA-E , HLA-G] [13].

Moreover, the inflammatory processes within the tumor microenvironment promote the aggressive phenotype in ERα+ breast cancers. ERα and NF-κB activated by estrogens and proinflammatory mediators promote breast cancer cell survival, proliferation, and drug resistance. Crosstalk between ERα and NF-κB is context-dependent and can result in synergistic activation or mutual suppression [14].

Macromolecular studies suggested that HLA-G expressed by tumors contributes to the evasion of immunosurveillance as NF-κB may regulate HLA-G expression to be reflected as a hallmark of cancer [15, 16]. It inhibits the function of immune effector cells and APC by binding to its receptors ILT-2, ILT-4, and KIR2DL4, thereby blocking the cytolytic function of T lymphocytes and NK cells via ILT2 and their proliferation via ILT2 and ILT4 [17].

The molecular mechanisms that upregulate HLA-G expression in tumor cells are complex. They involve several factors including epigenetic control of the HLA-G promoter activation and a variety of environmental factors such as hypoxia, stress, hormones, certain cytokines, and viral infection [18]. The functional relevance of HLA-G varies according to its expression by tumor cells or tumor-infiltrating cells [19]. When expressed by tumor cells, it constitutes an efficient way for escaping from immunosurveillance in view of its immunosuppressive properties. In contrast, HLA-G expression could be beneficial when it is expressed by tumor-infiltrating cells which bear ILT2, an inhibitory receptor known to interact with HLA-G [20]. Therefore, its effect on immune cells could also consist of limiting their Th1 cytokines secretion and subsequently chronic inflammation associated with tumor growth [21]. Accordingly, the aim of the current study was to verify the potential role of sHLA-G and its relation to NF- κB in patients with breast cancer.

2. Subjects and Methods

This study was conducted on 45 female members, participants were categorized into three groups: a control group consisting of 10 healthy individuals, a benign group comprising 10 patients with benign breast lesions, and a malignant group of 25 patients diagnosed with breast cancer at stage II or higher. Informed written consent was obtained from all participants prior to their inclusion in the study, the study was approved by the Ethics Committee of the Medical Research Institute, Alexandria University. The patients were randomly selected from those admitted to the Department of Experimental and Clinical Surgery and the Department of Cancer Management and Research at the Medical Research Institute, University of Alexandria.All participants were subject to detailed history investigation, clinical examination, and routine laboratory and diagnostic investigations.

2.1 Clinical Laboratory Investigations

These include liver function tests as serum aspartate aminotransferase (AST) [22] and alanine aminotransferase (ALT) [22], kidney function tests as serum urea and creatinine, and a complete blood picture [23].

2.2 Pathological Examination

The pathological diagnosis of breast lesions was based on fine needle aspiration or tissue biopsy, which included either excision biopsy or mastectomy. Tumor tissue sections were processed and examined in the Pathology Department of the Medical Research Institute, University of Alexandria. The specimens were stained with hematoxylin and eosin (H&E) for histopathological evaluation and tumor grading. Additionally, paraffin-embedded sections of postoperative tissue biopsies were stained to assess estrogen receptor (ER) and progesterone receptor (PR) status [24].

2.3 Immunological Examination

2.4.1 Sample Preparation

Peripheral blood samples were collected from all subjects prior to any therapeutic or surgical intervention. For serum preparation, 2 mL of blood were collected into uncoated vacutainers and left to clot at 37°C for 30 to 60 minutes, followed by an additional hour at 4°C to allow clot retraction. Samples were then centrifuged at 1800 rpm for 10 minutes, and the resulting sera were aliquoted and stored at −80°C until use [25].

2.4.2 Peripheral Blood Mononuclear cells (PBMCs) Isolation

Cells were isolated from 4 mL of heparinized venous blood using density gradient centrifugation over Ficoll-Hypaque (1.077) [26]. The procedure involved diluting the blood with an equal volume of sterile normal saline and gently mixing it in sterile Falcon tubes. This diluted sample was carefully layered over Ficoll-Hypaque in a 2:1 ratio and centrifuged at 1800 rpm for 45 minutes at room temperature. The mononuclear cell layer at the interface was then collected into a new sterile tube. PBMCs were washed first with normal saline by centrifugation at 1200 rpm for 10 minutes, followed by a second wash using RPMI-1640 tissue culture medium containing 2 mmol/L L-glutamine. Finally, the PBMCs were resuspended in 1 mL of RPMI-1640 for subsequent analysis.

Cells were counted using hemocytometer (Neubauer, Germany). Number of cells in one mL was calculated according to the following equation:

Number of cells/mL = Number of counted PBMCs x 5 x 10^4

The final concentration of the cells was adjusted to $2x10^6$ cells/mL that was calculated from original pool

2.4.3 Lysis of PBMCs

The lysis of peripheral blood mononuclear cells (PBMCs) was performed using the freeze-thaw method [27], a regularly utilized technique for lysing bacterial and mammalian cells. This method based on repeated cycles of freezing and thawing, causing the cells to swell and rupture due to ice crystal formation during freezing and contraction during thawing. Efficient lysis typically requires two to three cycles.

Cell pellets were first resuspended in 1 mL of phosphate-buffered saline (PBS, pH 7.2). The suspension was then exposed to three freeze-thaw cycles, this includes freezing at ≤−20°C and rapid thawing at 37°C with gentle mixing. After the final cycle, the lysed cell mixture was centrifuged at 1500 rpm for 10 minutes to remove any remaining cellular debris. The resulting supernatants containing the cell lysate were collected and stored at −80°C until further use.

2.4.4 Tissue Homogenization

Tissue samples obtained from surgically removed benign breast lesions, malignant tumors, and normal breast tissue adjacent to malignant lesions were collected on ice and immediately stored at −80°C until further use.

Tissues were firstly rinsed thoroughly in cold phosphate-buffered saline (PBS, 0.02 mol/L, pH 7.2) to remove any excess blood and then weighed. The tissues were shredded into small pieces and then homogenized in cold PBS using 1:3 ratio of weight-to-volume (tissue:buffer) with a glass homogenizer on ice. The homogenates were subjected to two freeze-thaw cycles to enhance cellular disruption. The samples were then centrifuged at 5000 rpm for 5 minutes. The supernatants were then immediately collected and stored at −20°C for subsequent analyses.

2.4.5 Assessment of HLA-G Level

HLA-G concentration was determined in both serum and tissue homogenate samples from all participating females using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science Inc., Human HLA-G ELISA Kit, Wuhan, China) according to manufacturer's instructions.

A series of seven standard dilutions were prepared using 0.5 mL of standard diluent per tube. Serum samples were diluted 1:50 with 0.02 M phosphate-buffered saline (PBS, pH 7.2) by mixing 10 µL of serum with 450 µL of PBS. Assay Diluent A and B were each diluted with 6 mL of distilled water to obtain 12 mL of working solution. Detection Reagents A and B were diluted to a 1:100 working concentration using their assay diluents. Additionally, 20 mL of concentrated Wash Solution was diluted with 580 mL of distilled water to prepare 600 mL of Wash Buffer.

All reagents and samples were brought to room temperature (18–25°C) for 1 hour. Then, 100 µL of each standard concentration, blank, and sample (diluted serum and tissue homogenate) were added to the designated wells. The plate was covered and incubated at 37°C for 2 hours. After incubation, the contents of each well were gently aspirated without washing. Next, 100 µL of Detection Reagent A working solution was added to each well, followed by another 1-hour incubation at 37°C.

Following incubation with Detection Reagent A, the wells were washed three times using 200 µL of Wash Solution per well, allowing 1–2 minutes per

wash. After the wash, 100 µL of Detection Reagent B working solution was added to each well, and the plate was covered and incubated in the dark at 37°C for 30 minutes. The washing step was three times as previously described.

Substrate solution 90 µL was added to each well, and the plate was incubated at 37°C for 15–25 minutes. The reaction was terminated by adding 50 µL of Stop Solution to each well. The optical density (OD) of each well was measured within 15 minutes using a microplate ELISA reader set at 450 nm.

The concentrations of HLA-G in the samples were calculated based on a standard curve constructed from the optical densities of the serially diluted standards. For serum samples, the final HLA-G concentration was obtained by multiplying the measured value by the dilution factor (×50).

2.4.6 Assessment of NF-κB Level

The level of nuclear factor kappa B (NF-κB) was measured in peripheral blood mononuclear cell (PBMC) lysate and tissue homogenate samples collected from all female participants in the study. The measurement was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Uscn Life Science Inc., Human Total NF-κB ELISA Kit, Wuhan, China) according to the manufacturer's instructions [28]. The assay used quantitative sandwich ELISA technique to determine the total expression level of NF-κB, representing the combined cytosolic and nuclear protein fractions.

The standard was reconstituted with 1.0 mL of Standard Diluent and allowed to sit at room temperature for 10 minutes before gentle shaking to avoid foaming. The concentration of the stock standard was 40 ng/mL, which was then diluted to 10 ng/mL to serve as the highest standard. All reagents and samples were equilibrated to room temperature (18–25°C) before initiating the assay.

For Assay;100 µL of each standard concertation, blank, and sample (tissue homogenates and PBMC lysates) were added to their respective wells in the ELISA plate. The plate was covered and incubated at 37°C for 2 hours. After incubation, the contents of the wells were carefully aspirated without washing.

Next, 100 µL of the working solution of Detection Reagent A was added to each well, and the plate was covered and incubated for 1 hour at 37°C. After this incubation, the wells were washed three times with 200 µL of Wash Solution per well, allowing 1–2 minutes per wash. Subsequently, 100 µL of Detection Reagent B was added to each well, followed by a 30-minute incubation in the dark at 37°C. The aspiration and wash step were repeated three more times as previously described.

Following the washing, 90 µL of Substrate Solution was added to each well, and the plate was incubated for 15–25 minutes at 37°C. The reaction was stopped by adding 50 µL of Stop Solution to each well. Finally, the optical density (OD) of each well was measured within 15 minutes using a microplate ELISA reader set to 450 nm. The concentrations of NF-κB in the samples were calculated based on a standard curve generated from the optical density values of the standard dilutions.

2.4.7 Statistical Analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Quantitative data were described using mean, standard deviation and range.

The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test, also Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests were applied. If the data were abnormally distributed, non-parametric tests were used.

For abnormally distributed data, Mann-Whitney Test (for data distribution that was significantly deviated from normal) was used to analyze two independent populations. If more than two populations were analyzed Kruskal Wallis test to be used.

For normally distributed data, Correlations between two quantitative variables were assessed using Pearson coefficient. For abnormally distributed data, Correlations between two non-parametric variables were assessed using Spearman coefficient.

Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

Agreement of the different predictive with the outcome was used and was expressed in sensitivity, specificity, positive predictive value, negative predictive value and accuracy. Receiver operating characteristic curve (ROC) was plotted to analyze a recommended cut-off, the area under the ROC curve denotes the diagnostic performance of the test. Area more than 50% gives acceptable performance and area about 100% is the best performance for the test.

3. Results

3.1 Demographic Data

The current study was conducted on a total of randomly chosen 45 females who were divided into three groups: Control Group; ten normal healthy control females, Benign Group; ten patients with benign breast lesions.

Malignant Group : Twenty-five patients with breast cancer (stage II or more).

The age of included females ranged between 22–65, 17–53, 33–68 years in Control, Benign, and Malignant groups, with mean ± SD= 39.4± 3.85, 30.70± 1.77, 51.72±1.68 years, respectively (Figure 1).

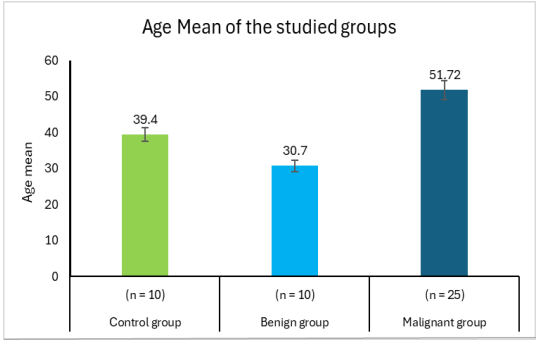

Figure 1 Age data of the studied groups

3.2 Biochemical Test for the Studied groups

3.2.1 Liver Function Tests: AST and ALT

For aspartate aminotransferase (AST), the malignant group showed a range of 12.0–47.0 IU/L with a mean value of 21.08 ± 7.70 IU/L. The benign group exhibited a narrower range (15.0–30.0 IU/L), with a slightly higher mean of 22.40 ± 5.34 IU/L, while the control group had values between 14.0–32.0 IU/L and a mean of 21.90 ± 6.24 IU/L. AST values were comparatively similar across all groups.

Alanine aminotransferase (ALT), for the malignant group the range was 12.0–48.0 IU/L and a mean value of 19.44 ± 8.97 IU/L. The benign group ranged from 11.0–21.0 IU/L with a mean of 17.1 ± 4.98 IU/L, whereas the control group ranged from 16.0–29.0 IU/L and showed a higher mean of 21.50 ± 6.31 IU/L. These findings suggest that ALT levels were slightly lower in the benign group compared to the control and malignant groups figure 2.

3.2.2 Renal Function Tests: Serum Urea and Creatinine

Regarding serum urea, the malignant group showed the highest range (17.0–44.0 mg/dL) with a mean of 26.8 ± 6.81 mg/dL. The benign group ranged between 15.0–33.0 mg/dL with a mean of 24.9 ± 5.57 mg/dL, and the control group ranged from 15.0–32.0 mg/dL with a mean of 25.1 ± 6.05 mg/dL. These values indicate mild variations among the groups, with slightly elevated levels in the malignant group.

Serum creatinine levels remained fairly consistent across all groups. The malignant group had a range of 0.6–1.1 mg/dL with a mean of 0.87 ± 0.13 mg/dL. The benign group ranged from 0.6–1.2 mg/dL with a mean of 0.84 ± 0.16 mg/dL, while the control group ranged from 0.7–1.1 mg/dL with a mean of 0.85 ± 0.15 mg/dL. These findings suggest preserved renal function in all studied groups (Figure 2).

3.2.3 Haematological Parameters: WBCs Count and Haemoglobin

The white blood cell (WBC) count showed a wide variation in the malignant group (8.5–17.6 $\times 10^3/\mu L$), with a mean of 6.68 ± 2.98 $\times 10^3/\mu L$, suggesting possible systemic inflammation or tumor-related immune response. The benign group had a lower range (2.7–8.3 $\times 10^3/\mu L$) with a mean of 5.41 ± 1.55 $\times 10^3/\mu L$, while the control group exhibited a range of 4.3–9.5 $\times 10^3/\mu L$ and a mean of 6.11 ± 1.56 $\times 10^3/\mu L$.

Haemoglobin levels were relatively consistent across all groups. The malignant group ranged from 9.6–13.2 g/dL with a mean of 11.63 ± 1.12 g/dL. The benign group had a range of 9.8–12.4 g/dL and a mean of 11.62 ± 0.95 g/dL, while the control group ranged from 10.9–12.5 g/dL with a mean of 11.73 ± 0.78 g/dL. Overall, these results indicate comparable haemoglobin levels, though slightly lower minimum values were noted in the malignant and benign groups (Figure 2).

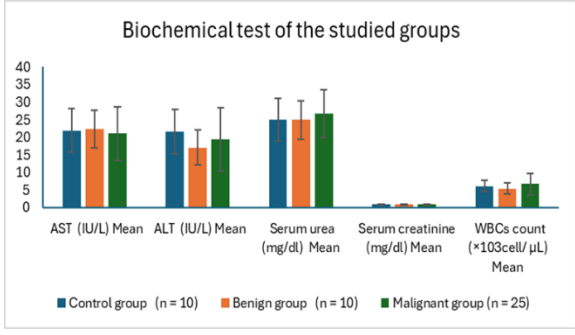

Figure 2 Biochemical tests of the studied

groups

3.3 Clinicopathological Analysis

The clinical data represented in figure 3 provides comparison among control, benign, and malignant groups regarding family history and menstrual status. The number of individuals with a positive family history was negligible in the control and benign groups, while only a small percentage was noted in the malignant group, suggesting limited genetic predisposition reported across the sample.

Most individuals in the control and benign groups had regular menstrual cycles, at 70% and 80% respectively. Alternatively, the percentage of women with regular menstruation in the malignant group, was significantly lower (around 40%), with a notable increase in individuals who had reached menopause (approximately 55%). A smaller percentage of women from the malignant group also suffered irregular cycles (under 10%). These findings highlight a shift in menstrual patterns among malignant cases, particularly a higher prevalence of menopause, which may correlate with age or hormonal factors associated with malignancy.

Pathological analysis of the malignant group (n = 25) showed that the major histological type was invasive ductal carcinoma (IDC) 84% of individuals (21 patients). Invasive lobular carcinoma was detected in 12% (3 patients), while only 4% (1 patient) have bifocal mucoid carcinoma, demonstrating that IDC is the most frequent breast cancer subtype in this cohort.

TNM staging revealed that 36% of the patients (9 cases) were categorised as stage II A, 24% (6 cases) as stage II B, 28% (7 cases) as stage III A, and the residual 12% (3 cases) was classified as stage III C. Stage II and III disease was detected in that the majority of patients as they were diagnosed at a relatively advanced stage (Figure 3).

Further grading of the 21 IDC cases showed that the majority (85.7%, 18 patients) had tumors grade II, while the remaining 14.3% (3 patients) were grade III. This suggests a tendency toward moderately differentiated tumors in most cases (Figure 3).

In terms of vascular invasion, 80% of the patients (20 cases) have vascular invasion, while 20% (5 cases) were negative, highlighting the aggressive nature of the disease in a significant proportion of cases.

Lymph node involvement was also evaluated, with 28.62% of the cases (89 nodes) have positive lymph node metastasis and 71.38% (222 nodes) were negative. The detection of nodal involvement in more than quarter of the patients underlines the risk of disease progression and metastasis in this population (Figure 3).

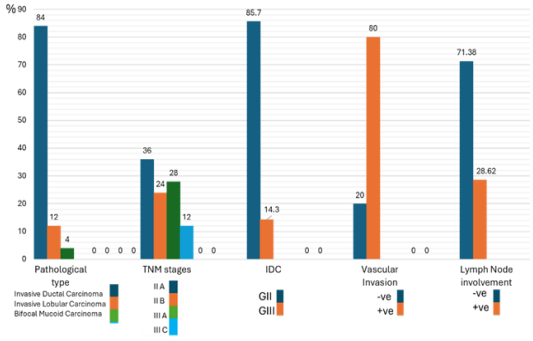

Figure 3 Pathological evaluation of the studied groups

3.4 Hormonal Receptor Expression

In the malignant group (n = 25), analysis of estrogen receptor (ER) and progesterone receptor (PR) expression revealed that the majority of patients (72%, 18 cases) were positive for both ER and PR (ER^+ PR^+). Discordant expression was observed in 20% of cases (5 patients) who were ER^+ PR^-, and in 4% (1 patient) who were ER^- PR^+. Only one case (4%) was negative for both receptors (ER^- PR^-), indicating a predominance of hormone receptor positivity in the malignant group (Figure 4).

Regarding the degree of ER positivity (n = 23), moderate expression (ER^{++}) was the most common, reported in 52.2% (12 patients), followed by weak expression (ER^+) in 30.4% (7 patients), and strong expression (ER^{+++}) in 17.4% (4 patients). This distribution suggests that most tumors exhibited moderate to strong ER expression, which is often associated with better response to endocrine therapy.

Similarly, analysis of PR expression intensity (n = 19) showed that more than half of the patients (52.6%, 10 cases) had weak PR

positivity (PR^{+}), while 42.1% (8 patients) exhibited moderate positivity (PR^{++}), and only one patient (5.3%) showed strong PR expression (PR^{+++}). These results demonstrate that PR expression, although generally present, tended to be lower in intensity compared to ER, which may influence the overall hormonal responsiveness and therapeutic planning.

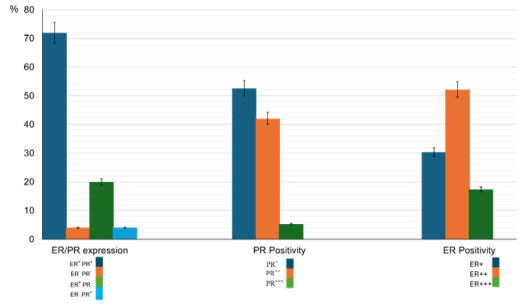

Figure 4 Estrogen and Progesterone Receptor Expression in Breast Carcinoma Patients

3.5 Immunological Markers: HLA-G and NF-κB

3.5.1 Tissue and Serum Levels of HLA-G

Tissue HLA-G expression levels was 0.05 to 0.16 ng/g tissue in homogenates of benign tissue (mean ± SD = 0.08 ± 0.05 ng/g tissue), and from 1.29 to 3.22 ng/g tissue in malignant tissue homogenates (mean ± SD = 2.42 ± 0.57 ng/g tissue), and from 0.49 to 2.80 ng/g tissue in peri-malignant tissue homogenates (mean ± SD = 1.45 ± 0.70 ng/g tissue).

Alternatively, benign tissue homogenate tHLA-G level was significantly elevated in both malignant and peri-malignant tissue homogenates ($p < 0.001$). Furthermore, tHLA-G levels were significantly higher in malignant tissues compared to peri-malignant tissues in the same 10 breast cancer patients ($p = 0.002$) (Figure 5).

Serum soluble HLA-G (sHLA-G) levels ranged from 7.42 to 11.98 ng/mL in the normal control group (mean ± SD = 8.99 ± 1.38 ng/mL), 7.42 to 11.74 ng/mL in the benign group (mean ± SD = 9.25 ± 1.47 ng/mL), and 7.42 to 15.70 ng/mL in the malignant group (mean ± SD = 10.83 ± 2.51 ng/mL).

Compared with the control group, the mean sHLA-G level was significantly increased only in the malignant group ($p = 0.041$), while no significant difference was observed between the control and benign groups ($p = 0.521$). The increase in the malignant group was also non-significant compared to the benign group ($p = 0.079$) (Table 1 and Figure 5).

In both benign and malignant groups, serum sHLA-G levels were significantly higher than their tissue (tHLA-G) counterparts ($p < 0.001$)

3.5.2 Tissue and Cell Lysates of NF-κB

Tissue NF-κB levels ranged from 0.83 to 5.54 ng/g tissue in benign tissues (mean ± SD = 2.12 ± 1.52 ng/g tissue), 1.13 to 16.65 ng/g tissue in malignant tissues (mean ± SD = 5.17 ± 3.71 ng/g tissue), and 1.28 to 5.01 ng/g tissue in peri-malignant tissues (mean ± SD = 2.49 ± 1.30 ng/g tissue).

Compared to benign tissues, the increase in NF-κB levels was significant in malignant tissues ($p = 0.003$) but not in peri-malignant tissues ($p = 0.173$). Compared with peri-malignant tissues, NF-κB levels were significantly higher in malignant tissues ($p = 0.014$).

NF-κB levels in cell lysates ranged from 0.60 to 1.47 ng/mL in the control group (mean ± SD = 1.02 ± 0.33 ng/mL), 0.65 to 1.85 ng/mL in the benign group (mean ± SD = 1.00 ± 0.38 ng/mL), and 2.15 to 5.58 ng/mL in the malignant group (mean ± SD = 2.88 ± 0.77 ng/mL).

The mean NF-κB level was significantly higher in the malignant group compared to both the control and benign groups ($p < 0.001$). No significant difference was observed between the control and benign groups ($p = 0.705$)

The mean tissue levels of NF-κB in both malignant and benign groups were significantly higher than their corresponding cell lysate levels ($p = 0.003$ and $p = 0.019$, respectively) (Figure 5).

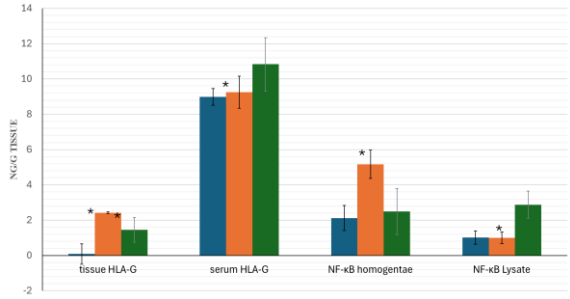

Figure 5. HLA-G and NF-κB expression levels

3.6 Association Between Clinicopathological Features and Serum sHLA-G Levels in Malignant Breast Cancer Patients

Serum levels of soluble HLA-G (sHLA-G) were analyzed in relation to various clinicopathological parameters among breast cancer patients. Patients aged below 52 years (n=12) had a slightly higher mean sHLA-G level (11.61 ± 2.80 pg/mL) compared to those aged 52 or older (n=13; 10.10 ± 2.07 pg/mL), although this difference was not statistically significant (p = 0.231). Similarly, no significant difference was found based on tumor size, with patients having tumors <60 mm (n=20) showing mean levels of 11.02 ± 2.54 pg/mL and those with tumors ≥60 mm (n=5) showing 10.06 ± 2.48 pg/mL (p = 0.415).

Regarding menstrual status, women with irregular cycles (n=2) showed the highest sHLA-G levels (14.26 ± 0.77 pg/mL), followed by those with regular menstruation (n=13; 11.46 ± 2.91 pg/mL), and menopausal patients (n=10; 9.82 ± 1.69 pg/mL), but without statistical significance (p = 0.283). When stratified by pathological type, patients with invasive lobular carcinoma (n=3) had the highest levels (13.45 ± 1.49 pg/mL) compared to those with invasive ductal carcinoma (n=21; 10.48 ± 2.48 pg/mL) and bifocal mucoid carcinoma (n=1; 10.44 pg/mL), with no significant differences (p = 0.132).

Within the invasive ductal carcinoma group, Grade II tumors (n=18) and Grade III tumors (n=3) exhibited comparable sHLA-G levels (10.48 ± 2.67 and 10.45 ± 1.08 pg/mL, respectively; p = 0.615). TNM staging also showed no significant correlation, with stage IIIA patients displaying slightly elevated levels (11.35 ± 2.24 pg/mL) compared to stage IIA (10.85 ± 2.65 pg/mL), IIB (10.41 ± 2.04 pg/mL), and IIIC (10.44 ± 4.51 pg/mL; p = 0.848).

Additionally, serum sHLA-G levels did not differ significantly based on lymph node involvement (p = 0.910) or vascular invasion (p = 0.135). Notably, patients without vascular invasion (n=5) had slightly higher sHLA-G levels (12.25 ± 2.49 pg/mL) compared to those with invasion (n=20; 10.48 ± 2.45 pg/mL). Analysis of hormone receptor status showed increasing sHLA-G levels with higher ER positivity, from 9.68 ± 1.35 pg/mL in ER^{+} to 12.94 ± 2.87 pg/mL in ER^{+++} cases (p = 0.190). A similar but non-significant trend was observed with PR expression: PR^{+} (11.70 ± 2.09 pg/mL), PR^{++} (10.18 ± 1.26 pg/mL), and PR^{+++} (11.90 ± 3.35 pg/mL; p = 0.491).

These findings suggest no statistically significant association between sHLA-G levels and clinicopathological variables, although certain trends—particularly with hormonal receptor expression—may need further investigation in larger cohorts.

Table 1. Comparative analysis of sHLA-G across demographic and tumor variables data in patients' group.

Clinicopathological parameters	**Serum levels of sHLA-G**	*P* value level of significance
Age	Mean ± SD.	
<52 (n=12)	11.61 ± 2.80	0.231
≥ 52 (n=13)	10.10 ± 2.07	
Tumor size	Mean ± SD	
<60 (n=20)	11.02 ± 2.54	0.415
≥ 60 (n=5)	10.06 ± 2.48	
Menstrual status	Mean ± SD	
Menopause (n=10)	9.82 ± 1.69	0.283
Irregular menses (n=2)	14.26 ± 0.77	
Regular menses (n=13)	11.46 ± 2.91	
Pathological types	Mean ± SD	
Invasive ductal carcinoma (n=21)	10.48 ± 2.48	0.132
Invasive lobular carcinoma (n=3)	13.45 ± 1.49	
Bifocal mucoid carcinoma (n=1)	10.44	
Invasive ductal carcinoma grades	Mean ± SD	
Grade II (n=18)	10.48 ± 2.67	0.615

Grade III (n=3)	10.45 ± 1.08	
TNM stages	Mean ± SD	
II A (n=9)	10.85 ± 2.65	0.848
II B (n=6)	10.41 ± 2.04	
III A (n=6)	11.35 ± 2.24	
III C (n=3)	10.44 ± 4.51	
Lymph node involvements	Mean ± SD	
Show tumor invasion (n=9)	10.86 ± 2.64	0.910
Show tumor no invasion (n=16)	10.82 ± 2.52	
Vascular invasions	Mean ± SD	
-ve (n=5)	12.25 ± 2.49	0.135
+ve (n=20)	10.48 ± 2.45	
ER degree of positivity	Mean ± SD	
+ve (n=7)	9.68 ± 1.35	0.190
++ve (n=12)	11.53 ± 2.65	
+++ve (n=4)	12.94 ± 2.87	
PR degree of positivity	Mean ± SD	
+ve (n=10)	11.70 ± 2.09	0.491
++ve (n=8)	10.18 ± 1.26	
+++ve (n=1)	11.90 ± 3.35	

4. Discussion

The present study investigated clinical, biochemical, hematological, pathological, and immunological profiles in breast cancer patients, individuals with benign breast lesions as compared to healthy controls. This study investigates breast cancer progression and immune modulation, particularly through the evaluation of liver and renal function, inflammatory markers, hormone receptor status, and immune-related molecules such as HLA-G and NF-κB.

Demographic and Clinical Findings

Breast cancer mean age was significantly higher than those in the benign and control groups. This agrees with previous literature indicating that increasing age is a risk factor for breast cancer development [29]. Menstrual irregularities and menopause prevalence were more frequent in malignant cases, which indicates the effect of hormonal changes on breast cancer risk and progression [30].

Biochemical and Hematological Parameters

Liver function enzymes (AST and ALT) did not differ significantly between groups, indicating insignificant hepatic involvement or toxicity. No variation was detected in Renal markers (urea and creatinine), suggesting normal kidney function. However, slightly elevated WBC counts in the malignant group indicated systemic inflammation or tumor-induced immune modulation [31]. Haemoglobin levels were comparable across groups, although slightly lower values in the malignant and benign groups may suggest subclinical anaemia or tumor-related inflammation [32].

Pathological Features and Hormone Receptor Expression

Most tumors were of grade II and staged as II or III, indicating diagnosis at advanced disease stages. Vascular invasion and lymph node metastasis were observed in most of malignant cases indicating the aggressive nature and metastatic potential of the disease.

High prevalence of ER and PR positivity was detected, especially ER^+/PR^+ co-expression. These findings are in agreement with previous clinical studies suggesting hormone receptor-positive tumors generally respond well to hormonal therapy with better prognosis [33]. Remarkably, ER expression was higher than PR, this was previously reported in hormone-responsive cancers [34].

Immunological Markers: HLA-G and NF-κB

Target findings of this study was to find the distinct expression pattern of HLA-G in both tissue and serum samples. Tissue HLA-G (tHLA-G) levels were significantly higher in malignant and peri-malignant tissues as compared to benign lesions. Elevated levels in malignant tissues suggested a role of tHLA-G in immune evasion mechanisms [35]. Similarly, serum HLA-G (sHLA-G) was significantly higher in the malignant group as compared to controls, suggesting it to be used as a circulating biomarker for breast cancer [36].

NF-κB was significantly upregulated in malignant tissues and PBMC lysates confirming its role as a transcription factor involved in inflammation and tumor progression [37]. The observed abundance of NF-κB is in accordance with its known role in promoting tumor proliferation, and resistance to apoptosis [38]. The correlation between increased NF-κB expression and malignancy supports the concept of a protumorigenic inflammatory environment in breast cancer [39].

Association Between sHLA-G and Clinicopathological Variables

No statistically significant associations were observed between serum sHLA-G levels and clinicopathological parameters, specific expression patterns were documented. Higher sHLA-G levels were observed in younger patients, those with irregular menstruation, and higher hormone receptor expression, although not significance. These trends suggest immunomodulatory or hormonal influences that merit further exploration in larger cohorts.

Implications and Future Directions

The elevated levels of sHLA-G and NF-κB in breast cancer patients underscore their potential utility as biomarkers for disease progression and immune escape. The interplay between hormone receptor status and immune regulation suggests a complex relationship between endocrine and immune pathways in breast cancer biology. Future studies should aim to validate these findings in larger populations and investigate whether combining immune and hormonal biomarkers can enhance diagnostic and prognostic accuracy.

5. Conclusion

This study investigates the clinical and immunological profile among healthy individuals, patients with benign and malignant breast cancer. Elevated tissue and serum levels of HLA-G and NF-κB in breast cancer patients underline their contribution in tumor-induced immune modulation and inflammation-driven development. While no significant correlations were found between serum HLA-G levels and most clinicopathological variables, notable trends suggest potential relevance to hormone receptor status and tumor subtype.

Our findings showed that both HLA-G and NF-κB are involved in breast cancer immune evasion and can be used as a diagnostic and prognostic biomarker. Further studies on large number of patients are required to validate their clinical effectiveness and to confirm their prognostic role. Integration of such immunological markers with standard histopathology and hormonal analysis could improve personalized therapy in breast cancer treatment.

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Timeline of Publication

Received	**Date:**	1	May	2025
Revised	**Date:**	28	May	2025
Accepted	**Date:**	4	June	2025
Published	**Date:**	30	June	2025